Contributions to Entomology 73 (2) 2023, 195–199 | DOI 10.3897/contrib.entomol.73.e109843

CONTRIBUTIONS
TO ENTOMOLOGY

SENCKENBERG

Research Article

Functional morphology of the genitalia of *Rhyacophila lezeyi* Navas, 1933 (Trichoptera, Rhyacophilidae)

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Academic editor: Astrid Schmidt-Kloiber | Received 27 July 2023 | Accepted 1 November 2023 | Published 23 November 2023

Abstract

We collected seven fixed pairs of *Rhyacophila lezeyi* during mating copulation and observed four different states of copulation. We investigated the underlying mechanism for the variations in the morphology of each copulating device, based on the copulation state. Notably, the male anal sclerites underwent considerable changes from the early stages of copulation. Initially, the female segment VIII approached the male anal sclerites, which were pushed downwards by the female VIII and IX segments, extending from IX. With the progression of mating stage, the distended end of the female's segment VIII covered the anal sclerites, pushing them further down. The male parameres were initially folded in bellows-like shapes under the aedeagus before copulation initiation. However, as the copulation stage advanced, they extended to about 3.2 times of their original length. Distended ends of both parameres adhered to the position of the spiracles at the anterior margin of the lateral part of the female's VII abdominal sternite. The attachment site was the external surface of the hair bulb of the male parameres. During the middle stage of copulation, movements involving the opening and closing of the male gonopods started. The gonoslylus made strong contact with the female's abdominal segment VIII, resulting in the deformation of segment VIII due to the contact pressure.

Key Words

anal sclerites, copulatory organ, female segment VIII, fixed pairs, mating copulation, parameres

Introduction

The identification of species is often based on the morphology of the copulatory organ, which is the most extensively studied morphological trait in caddisflies. The diversity of morphological characters of copulatory organs is well recognised in Trichoptera (Holzenthal et al. 2007). Although several hypotheses have been proposed as the mechanism for the diversity in copulatory organs (Eberhard 1985, 2010; Arnqvist 1997; Simmons 2014), the underlying reasons for tremendous morphological variations amongst species and families remain unclear. To understand the reason behind the evolution of these intertaxonomic differences in the shape of the copulatory apparatus, it is crucial to conduct a detailed investigation of the function of these apparatus during mating copulation.

While discussing the ground plan and basic evolutionary trends of male copulatory organs in caddisflies, Ivanov (2005) stated that using not only comparative morphological and historical (fossil), but also functional approaches is important in solving the evolutionary questions of copulatory organ morphology. He indicated that data on the function of male and female reproductive organs are scarce and more data showing the inter-sex structural relationships, such as the lock-and-key mechanisms, are indispensable (Sota and Kubota 1998). These relationships

* The paper is part of 17th International Symposium on Trichoptera, Edited by Simon Vitecek, Astrid Schmidt-Kloiber, Wolfram Graf, Hans Malicky.

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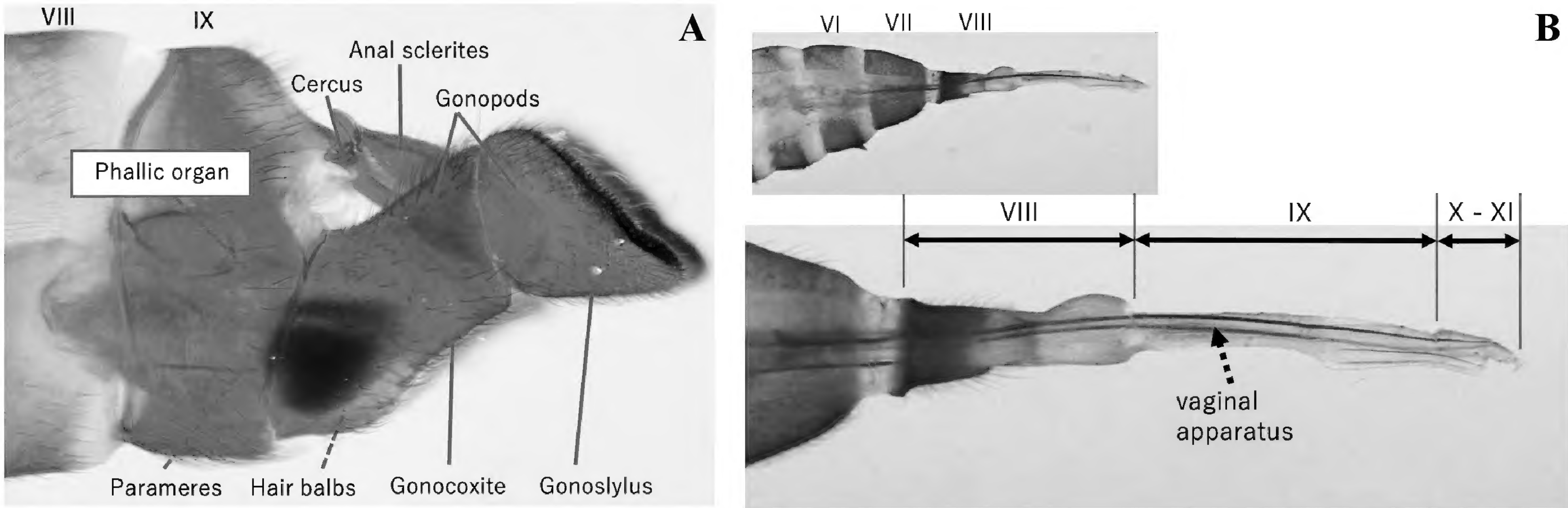

Figure 1. *Rhyacophila*: copulatory organ of the *acropedes* group, along with the names of its morphological characters. **A.** Male *R. articulata*; **B.** Female *R. lezeyi*.

should be evaluated by comprehensively observing the mating behaviour.

It is not difficult in itself for us to find mating caddisflies. We can often find mating pairs, especially in the diurnally active rhyacophilid caddisfly. However, it is difficult to observe pre-mating behaviour or examine how the copulatory apparatus functions during mating, even if many mating individuals can be seen. Parallelly-positioned females and males perform the so-called "mating turn", in which their heads face in the opposite directions, as soon as their respective abdominal ends are in contact. After the mating turn, the abdominal ends of the female and male are completely hidden under their respective wings, making it difficult to directly observe the way copulation proceeds and function of various parts of the copulatory apparatus. A reasonable way to observe the positioning and relationships between each part of the copulatory apparatus during the mating copulation is by capturing mating pairs at the copulation. In this study, rhyacophilid caddisfly pairs were frozen and fixed during the mating copulation in the field and the copulating state was comprehensively examined to clarify how the various parts of the copulation apparatus were related to each other between pairs.

Materials and methods

Rhyacophila lezeyi Navas, 1933 belongs to the *R. acropedes* species group (Ross 1956) and is one of the most prevalent species of Japanese rhyacophilid caddisflies. It is commonly found in the middle to upper reaches of rivers and has a wide distribution, spanning from the Honsyu, Shikoku to Kyushu Regions in Japan (Hattori 2005). During late spring to summer, adults are abundantly present and can be observed on vegetation, such as shrubs, along the riverside banks, where their larvae are also present. The type locality of *R. lezeyi* is Kofu, Yamanashi Prefecture, Japan (Kuranishi 2016).

Adults of *R. lezeyi* were collected on 8 June 2021 from Kosuge-gawa (35°45'16"N, 138°53'57"E), a mountain stream in Kosuge-mura, Yamanashi Prefecture in central Japan. As copulating pairs of the rhyacophilid caddisflies can be easily separated from each other due to external stress or disturbance, we took the following steps to preserve them in a mating state: 1) drop the mating pair very gently into an insect net, 2) freeze it instantaneously, with a cooling spray (the active ingredients are listed as HFO-1234ze and DME), 3) wrap the mating individuals in Kimwipe (Kimberly Clark Corp.), 4) soak the wrapped specimens in boiling water for a few seconds, 5) put them in plastic tubes filled with 75% ethanol, 6) take the samples to the laboratory, 7) observe the appearance of the mating state and 8) detach the abdomen from the body and treat it with 5% potassium hydroxide for transparency. Mating pairs and reproductive organs were dissected under a stereomicroscope and micrographs were taken with a digital camera attached to the stereomicroscope.

Fig. 1 shows the fundamental structure of the copulatory organ of the *Rhyacophila acropedes* species group. Males possess prominent large gonopods and anal sclerites that move up and down. Parameres are folded in bellows-like shape and do not extend beyond the gonopods and have distinct hair bulbs at their apices. Phallic organs are small and concealed by the segment IX and gonopods. The female abdominal terminalia are externally visible up to the segment VIII under normal conditions, but the narrow membranous portion is extended during copulation. In rhyacophilid caddisfly, the terms aedeagus, preanal appendage, and inferior appendage are traditionally used to describe the morphology of genitalia; however, in this paper, these terms were replaced by the terms phallic organ, cercus, and gonopods, respectively.

Results

Seven pairs of *R. lezeyi* were collected in a copulating state that had completed the mating turn. The mating status of each pair was classified into four states as follows. State A (four of the seven pairs) was defined when the mating had been dissolved and the pairs were separated from each other (Fig. 2A, B). The common characteristics of these pairs were the non-elongated female VIII

segment, elongated male parameres compared with those before mating and their tips not exceeding the gonopods. Inside the parameres, a white gel-like secretion was observed, which became transparent after alkaline treatment. The anal sclerites were in the same state as those before mating and the gonostylus was slightly narrowed inwards, but not closed. State B (one of the seven pairs) was defined when the pair was fixed in the mating state (Fig. 2C, D). Female VIII and IX segments were elongated and directed medially towards the male copulatory organs. Male parameres were elongated, but remained in a state similar to State A. However, they did not contact

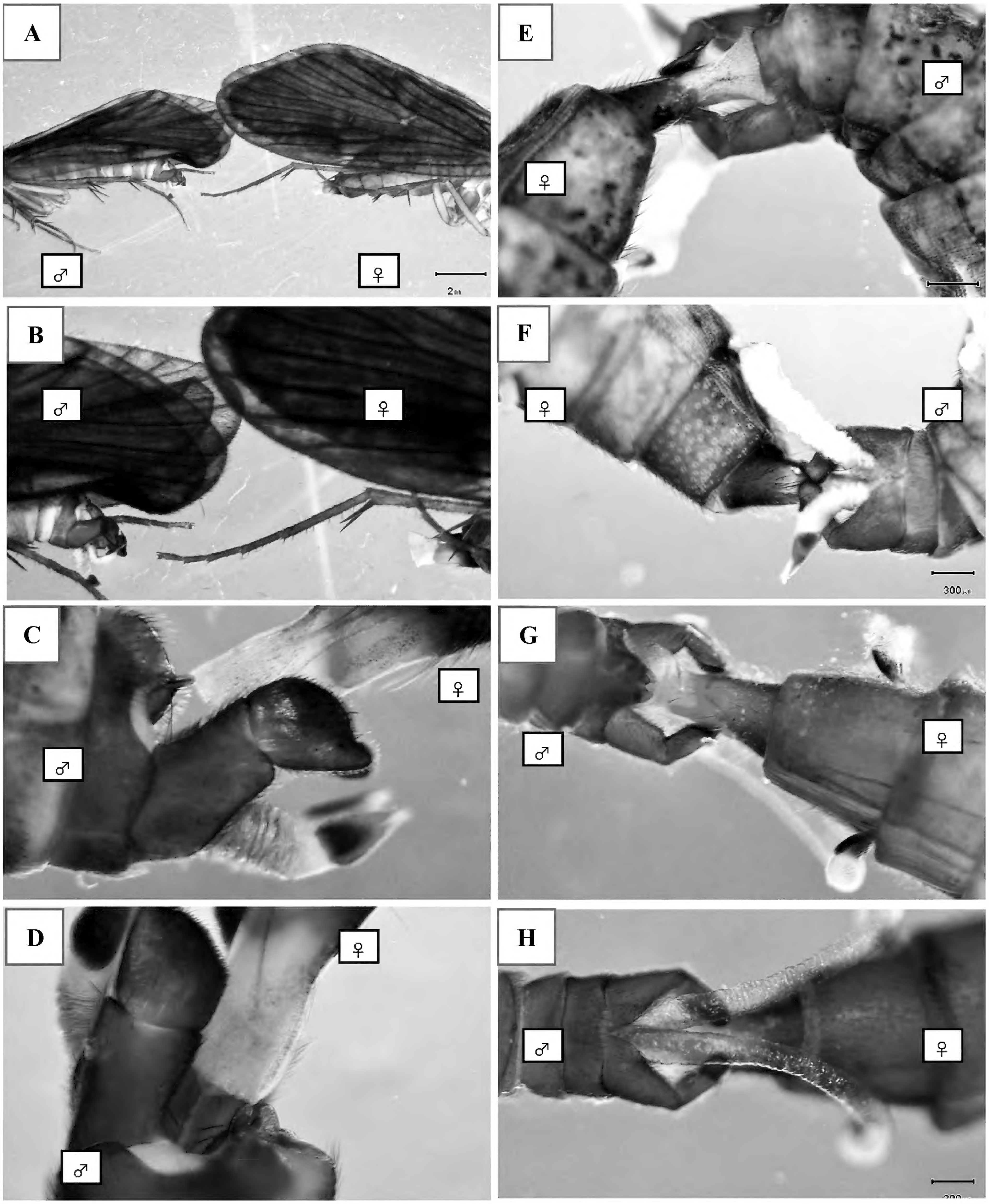

Figure 2. Four mating states of a pair of *R. lezeyi* (after completing the mating turn). **A, B.** State A; **C, D.** State B; **E, F.** State C; **G, H.** State D. Lateral view; **A–C.** Dorsal view; **E, G.** Ventral view; **F, H.** Male, inverted, lateral, obliquely from above; **D, C–H.** Wings have been removed.

the female's abdomen and their gonopods did not contact the female's abdominal segment. The anal sclerites were strongly bent downwards and in contact with the female's abdominal end. State C (one of the seven pairs) was defined when the pair was also fixed in the mating state (Fig. 2E, F). The anal sclerites were covered by the female VIII segment. The covering of the anal sclerites suggested a progression from State B, with a strong connection between the female ventral end and the male. Male parameres were markedly elongated, with a difference in the degree of elongation between the left and right sides. The right paramere was in contact with the anterior margin of the female VII segment and approximately 2.7 times longer than its length before mating. The hair bulb of the right paramere was also in direct contact with the female's body and white secretions were observed on its apical hair bulb. Male gonopods strongly clasped the female VIII segment. State D (one of the seven pairs) was defined when the pair was also fixed in the mating state (Fig. 2G, H). The gonopods tightly clasped the female VIII segment, which was under the strong pressure from the male gonopods, resulting in a heavily-concaved area of contact. Both male parameres were highly elongated approximately 3.2 times longer than their length before mating, reaching the anterior margin of the female VII segment and were in contact with the female's abdomen. White secretions were observed on the apical hair bulb of both parameres and the attachment site of the hair bulb was at the female's stigma. The elongated parameres were twisted 180° and in contact with the female's body outside the hair bulb. The main difference between pairs in State A (thought to have separated after copulation) and those remaining in copulation was the connection between the anal sclerites and the female's VIII segment extensions. Several states of the mating process from the extension of the parameres, while not touching the female's abdomen to the adherence of the two parameres to the female's abdomen, were observed in this study. The gonopods could clasp the female's abdomen only when the parameres were extended until they made contact with the abdomen.

Discussion

Although the paired specimens in this study were not strictly fixed at a specific time series after the male and female met and initiated copulation, the overall condition of the female VIII segment, anal sclerites, parameres and gonopods suggested a time elapsed during the progression from State A to State B to State C to State D. The present results clearly showed the sequence of mating behaviour of *R. lezeyi* after the mating turn. Based on these findings, it can be assumed that State A, when mating was not established, represented the very early stage of mating. It was found that, after the mating turn, genital locking does not first occur before the progression of mating situation. Before mating commenced, the male was observed to bend his abdomen in a U-shape and open and close his gonopods to approach the female. Despite conspicuous movement of the gonopods, the gonopodal lock was not completed during the early stages of copulation. Conversely, the state of the anal sclerites markedly changed immediately after the onset of the mating state following the mating turn. The female's abdominal end made direct contact with the male's anal sclerites, causing them to fold substantially in the downward direction. At this point, instead of the male phallic organ ejecting and approaching the female, the female's abdominal end extended and entered the male's copulatory apparatus. Before the mating began, the parameres were folded and stored under the phallic organ in a bellows-like shape. In the early stages of mating (States A and B), they were extended to about 1.5 times their normal length. In States C and D, the state of the parameres changed drastically, i.e. the parameres were elongated to approximately 2.7–3.2 times the length of the precopulating state and one or both of the parameres reached and contacted the female's abdomen.

Ivanov (2005) analysed the details of the copulation process of rhyacophilid caddisflies by monitoring the individuals in an observation cage on video. He considered that the "mating turn" is the final stage of copulation behaviour. In this study, careful examination of mating pairs fixed after the mating turn revealed that the connection of various parts of the copulatory apparatus was not complete even after the mating turn had occurred. This finding confirmed the empirical fact that the copulation of rhyacophilid caddisflies can be easily dissolved, even in pairs that have undergone a mating turn. Thus, we propose that the mating turn is merely an intermediate step in a series of mating behavioural movements for rhyacophilid caddisflies. Furthermore, we suggest that even after the mating turn, there are additional movements in various parts of the copulatory apparatus to complete their copulations. Although most of these movements are not directly observable yet owing to their being overlapped with their wings, further studies will help clarify the details of male and female copulatory organ movements through detailed monitoring.

Ivanov (2005) also noted that the males of rhyacophilid caddisfly positioned parallel to the female with bending the end of the abdomen into a U-shape and approaching the tip of the female's abdomen by abdominal ending. They repeated this approach several times, which was also observed in our sample. A male rhyacophilid caddisfly approached the female's abdominal terminal repeatedly with his genitalia being widely opened, which was accompanied by opening and closing movements. It was implied that the copulation behaviour appeared to be a male-led process. However, the examination of fixed copulating individuals revealed that copulation itself does not involve the insertion of the male into the female; rather, it is the

female who extends her abdominal end into the male's copulatory apparatus. The successful establishment of copulation relies on the extension the female's abdominal end. Therefore, it can be assumed that multiple approaches of the male were some types of behaviour aimed at inducing the extension of the female's abdominal end when it had not been extended.

Schmid (1970) fixed a male–female pair of *R. kanichka* from the Himalayas of Pakistan and successfully examined each part of the copulating state (Plate II, fig. 14 in his monograph). Schmid's figure is the sole illustration that presents the details of the copulating state of rhyacophilid caddisfly and connections between the copulatory organs. Schmid (1970) found that the connection between the terminal piece of the female vaginal apparatus and male anal sclerites, which was impaled within the male copulatory apparatus, was the strongest, whereas other connections were loose. In this study, it was confirmed that the extended abdominal end of the female was guided and clasped by the fine setae of the anal sclerites. However, the specific point within the male copulatory apparatus where the end of males and females are connected to form a strong connection could not be clarified. The issue of sperm delivery, which could not be confirmed in this study, remains a topic for future research. Regarding the function of the parameres, Schmid (1970) stated that the role of this part is not clear. In contrast, the present results clearly showed that the parameres function as the tactile system. It was observed that the parameres change their state during copulation, with the male twisting the parameres to bring the terminal hair bulb into contact with the female's abdomen. It is inferred that the parameres function not as the male's own sensory organ but as an active stimulator for the female.

Conclusion

Observations of fixed *Rhyacophila lezeyi* copulatory organs suggested the copulating state changed even after the mating turn. In *R. lezeyi*, the female abdominal terminal was inserted into the male anal sclerites. Anal sclerites may support and stabilise the copulating state. The parameres were markedly elongated and in contact with the female abdomen. In rhyacophilid caddisfly, the long abdominal terminal of the female is presumed to have a function not only in egg laying, but also in exploring the male's phallic organ during the early stages of copulation and in strengthening copulation.

Acknowledgements

We thank Yoshitaka Kamimura for his important technical advice and critical reading of the manuscript and thanks Vladimir D. Ivanov and John S. Weaver for kind advice and information. We thank Naotoshi Kuhara for providing micrographs of *R. articulata*.

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